Participation of GABA-benzodiazepine Receptor Complex in the Anxiolytic Effect of *Passiflora alata* Curtis (Passifloraceae)

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SUMMARY. *Passiflora alata* **Curtis is used in Brazilian folk medicine and also by pharmaceutical industry due to its tranquilizing properties. In this work, the central activity of an aqueous (AQ) and an hydroethanolic (HE) leaves extracts were evaluated in elevated plus maze, barbiturate sleeping time, open field and [³H]flunitrazepam binding assays. The only effect presented by AQ (300 mg/kg, p.o.) was on the barbiturate sleeping time, indicating a hypnotic effect. The HE extract (300 and 600 mg/kg, p.o.) also increased the barbiturate sleeping time and reduced the locomotor activity (at 600 mg/kg, p.o.), pointing to a sedative effect. In addition HE showed an anxiolytic-like effect (300 mg/kg, p.o.) in the elevated plus maze test which was blocked by flumazenil (6 mg/kg, i.p.). Nevertheless HE did not displace [³H]flunitrazepam binding to rat brain synaptosomes in concentrations up to 1000 μg/mL. As a conclusion, we showed that the anxiolytic effect of** *P. alata* **in mice depends on the dose and solvent used for the extract preparation, and this effect cannot be attributed to the direct activation of the central benzodiazepine site by the chemical constituents of the extract. It is possible that their metabolites or an indirect effect on benzodiazepine-GABA$_A$ receptor complex mediate the observed anxiolytic effect.**

INTRODUCTION

The *Passiflora* genus is widely distributed throughout tropical regions of the world and some species are used in folk medicine (as leaf infusion) for tranquilizing purposes [1]. The increasing number of studies on *Passiflora* reflects the potentiality of this genus as raw material for developing phytomedicines or as a source of anxiolytic compounds. *P. incarnata* L. is the species most commonly used in Europe being referred as a medicinal species with sedative and anxiolytic properties in different official publications [2,3], Pharmacopoeias [4] and scientific literature [5-10].
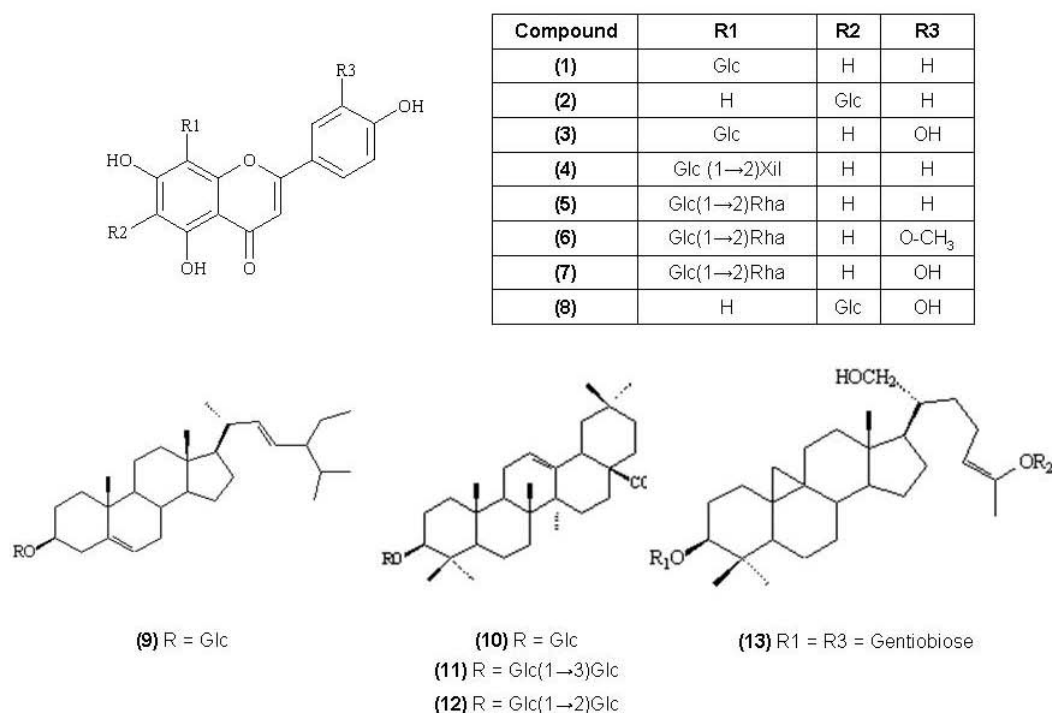
P. alata is the official species of the Brazilian Pharmacopoeia and it is present in several phytomedicines commercialized in Brazil as anxiolytic and sedative in the form of tinctures, elixirs and tablets [11]. Despite of this, there are few reports on its chemical composition, pharmacological properties and mechanism of action as follows. Previous studies showed that a *P. alata* fluid extract (75 and 150 mg/kg, i.p.) produced a hypnotic effect in rats [12]. An anxiolytic activity has also been reported for aqueous and hydroethanolic extracts (40%) of *P. alata,* at 100 and 150 mg/kg i.p. [13,14] and 640 mg/kg, p.o. [15] in rats and mice (100-600 mg/kg) [16]. Fiss *et al.* [17] reported the clinical efficacy and tolerability of some pharmaceutical preparations of *P. alata* for treating insomnia and mild anxiety. The safety of these preparations was also demonstrated in rodents [18].

Regarding the *P. alata* chemical composition, *C*-glycosidic flavonoids [19-21], saponins, as major constituents [22,23] (Fig. 1), and β-carboline alkaloids, in minor concentrations [12], have been reported.

Compound	R1	R2	R3
(1)	Glc	H	H
(2)	H	Glc	H
(3)	Glc	H	OH
(4)	Glc (1→2)Xil	H	H
(5)	Glc(1→2)Rha	H	H
(6)	Glc(1→2)Rha	H	O-CH₃
(7)	Glc(1→2)Rha	H	OH
(8)	H	Glc	OH

(9) R = Glc

(10) R = Glc
(11) R = Glc(1→3)Glc
(12) R = Glc(1→2)Glc

(13) R1 = R3 = Gentiobiose

Figure 1. Major constituents isolated from *P. alata* leaves: vitexin (**1**), isovitexin (**2**), orientin (**3**), 2"-xylosil-vi-texin (**4**), 2"-rhamnosyl-vitexin (**5**), 2"-rhamnosyl-scoparin (**6**), 2"-rhamnosyl-orientin (**7**), isoorientin (**8**), 3-O-β-D-glucopyranosyl-stigmasterol (**9**), 3-O-β-D-glucopyranosyl-oleanolic acid (**10**), 3-O-β-D-glucopyranosyl-(1→3)-β-D-glucopyranosyl-oleanolic acid (**11**), 3-O-β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl-oleanolic acid (**12**), 9,19-cyclolanostan-24Z-en-3β,21,26-trihydroxy-3,26-di-O-gentiobiose (**13**).

In this study we evaluated the anxiolytic and sedative effects in mice of oral single treatments with an aqueous and an hydroethanolic (70%) extracts obtained from *P. alata* leaves, and also investigated the involvement of the benzodi-azepine-GABA$_A$ receptor complex on the observed anxiolytic effects.

MATERIALS AND METHODS
Plant material
The aerial parts of *P. alata* were collected in the city of Canoas, State of Rio Grande do Sul, Brazil. The voucher specimen was deposited in the Herbarium of the Botanic Department of Universidade Federal do Rio Grande do Sul (ICN133727). The vegetal material was dried in a circulating air stove (35 °C) and triturated.

Preparation of plant extracts
Two different extracts were prepared. Air-dried powdered leaves of *P. alata* were extract-ed by reflux (plant:solvent, 1:10, w/v) for 1 h with H$_2$O to obtained the aqueous extract (AQ) and, separately, with EtOH:H$_2$O (70:30) to obtain the hydroethanolic extract (HE). The solvents were removed, separately, under vacuum at 40 °C to obtain both dried extracts, yielding 20% (AQ) and 25% (HE) in relation to dried plant.

Thin-layer Chromatographic Analysis (TLC)
Extracts were characterized by their flavonoid or saponin profiles using TLC according to Birk *et al.* [23].

Animals
For the behavioral experiments, adult male CF1 mice from Fundação Estadual de Produção e Pesquisa em Saúde (FEPPS, Porto Alegre, RS) were maintained in a local animal house in groups of 8 animals per plastic cage, at a temperature of 22 ± 2 °C, under controlled light/dark 12h cycle (lights on at 07:00 a.m.), with food and water *ad libitum* except at 6h before the experiments. Each animal was used just once. The number of animals ranged from 8 to 13 by groups. All the experiments were carried out between 12:00 a.m. - 16:00 p.m. In order to become familiar with the environment, the mice were placed in an experimental room two hs before the experiment. For the binding assay,

brains of adult rats from Universidade Federal do Rio de Janeiro were used. All experiments were approved by CONEP - Brazil (National Commission of Research Ethics) (2005512/01-2006) and performed according to the guidelines of The National Research Ethical Committee (published by National Heath Council - MS, 1998), which are in compliance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Drugs and dosages

The following drugs and doses were used: saline (0.9%), diazepam (2 mg/kg, v.o., Germed®), flumazenil (6 mg/kg, i.p., Cristalia®), sodium pentobarbital (40 mg/kg, i.p.), aqueous (AQ) and hydroethanolic (HE) extracts from *P. alata* (300 and 600 mg/kg). The animals were treated orally, by gavage, with a volume of 1 mL/ 100 g, 60 min before testing.

Elevated Plus Maze

This test is based on the model described by Lister (1987) [24]. Briefly, the apparatus consisted of two open arms (30x10 cm) and two closed arms (30x10x15 cm) perpendicularly disposed and linked by a central square (5x5 cm). The maze was 50 cm high. The animals received the different treatments and 60 min after were placed on the central square of the maze and the number of entries and time spent in open and closed arms were recorded during 5 min. Subsequently, the percentage of open arm entries and the percentage of time spent in the open arms were calculated for each animal. In another set of experiments, mice were treated with saline or flumazenil (6 mg/kg, i.p.) 30 min after the oral administration of the HE (300 mg/kg, p.o.).

Barbiturate sleeping time

Sixty min after drug administration, all animals received pentobarbital (40 mg/kg i.p.) and the parameters sleep latency and duration were recorded. The time elapsed between the loss and voluntary recovery of the righting reflex was recorded as sleeping time. A ceiling of 240 s was imposed in this measure.

Open field test

The apparatus consisted in a Plexiglas box with transparent walls and a black floor (45 x 30 x 30 cm) divided in 24 equal squares. Sixty min after drug administration, the number of cross-ings, rearings and groomings were recorded during a 5 min period.

In vitro experiments
Tissue preparation

Brain hemispheres were obtained from male Wistar rats sacrificed by decapitation. Briefly, tissues were homogenized in a Potter apparatus with a motor-driven Teflon pestle at 4 °C in 15 volumes of ice-cold 0.32 M buffered sucrose (pH 7.4) per gram of organ. After centrifuging at 1000 x g_{max} for 10 min, the supernatant was centrifuged at 48,000 x g_{av} for 20 min to obtain the crude synaptosomes that were resuspended in a buffered Krebs solution and stored at -80 °C until use.

[³H]Flunitrazepam binding

Synaptosomes (200 μg protein) were incubated at 4 °C for 90 min in a buffered Krebs solution containing different concentrations of hydroethanolic extract (10-1000 μg/mL) and 0.2 nM [³H]flunitrazepam (85 Ci/mmol, New England Nuclear Life Science Products, USA). After incubation, samples were rapidly diluted with 3 ml of ice-cold Krebs buffer and immediately filtered on glass fibre filters (GMF 3, Filtrak, Germany) under vacuum. Filters were then washed once more with 3 ml buffer, dried, and immersed in a scintillation cocktail and the radioactivity retained in the filters was counted with a Packard Tri-Carb 1600 TR liquid scintillation analyzer. Nonspecific binding was estimated in the presence of 5 μM unlabeled flunitrazepam [25].

RESULTS
Phytochemical screening

Flavonoids and saponins were detected as major constituents of both extracts which presented similar chemical profiles by TLC. Two spots were detected with characteristic flavonoid colour. Regarding the saponins, it was detected the presence of quadranguloside. The HE extract contained more apolar substances than the AQ extract.

Elevated plus maze

Mice treated with HE (300 mg/kg, p.o.) exhibited a significantly higher percentage of entries and time spent in the open arms (Fig. 2A). When considering the closed arms, such treatment induced a significantly reduced percentage of entries and time spent (Fig. 2B). Similar ef-
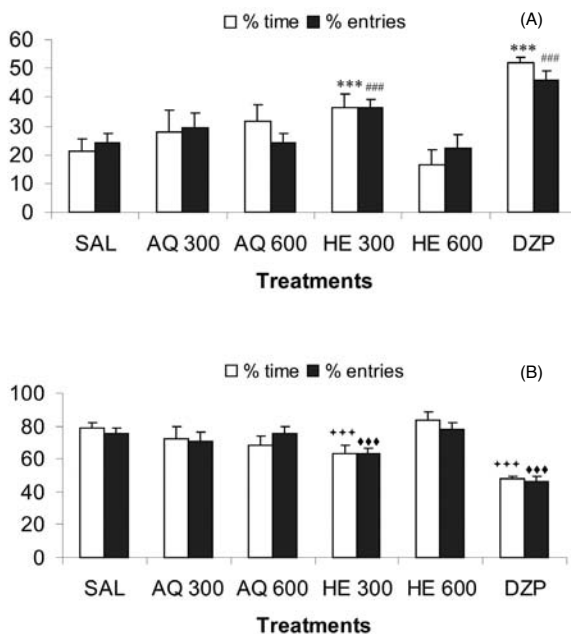
Figure 2. Effects of aqueous (AQ), hydroethanolic (HE) *Passiflora alata* extracts and diazepam (DZP) in the elevated plus maze model. Percentage of time spent and entries in the open (**A**) and closed (**B**) arms. Data are expressed as mean ± S.E.M. Statistical differences from control (SAL) were determined by ANOVA followed by Student-Newmann-Keuls test, *** $p<0,001$ ($F_{(5-65)} = 4,878$); ### $p<0,001$ ($F_{(5-65)}=5,019$); +++$p<0,001$ ($F_{(5-65)}=3,365$); ◆◆◆$p<0,001$ ($F_{(5-65)} = 4,572$). The number of animals by group ranged from 9 to11 by group.

Figure 3. Effect of flumazenil (FMZ 6 mg/kg, i.p.) on the effect of hydroethanolic extract (HE 300 mg/kg, v.o.) and diazepam (DZP 2 mg/kg, v.o.) in the elevated plus maze model. Percentage of time spent and entries in the open (**A**) and closed (**B**) arms. Data are expressed as mean ± S.E.M. Statistical differences were determined by Two Way ANOVA, *** $p<0,001$ when compared with the control group (SAL+SAL); ### $p<0,001$ when compared the groups with or without FMZ for the same treatment. The number of animals ranged from 10 to 13 by group.

fects were observed with diazepam, used as a positive control. Note that this effect was not observed when doubling the dose. On the other hand, no significant changes were observed when the animals were treated with AQ, at 300 and 600 mg/kg (Fig. 3).
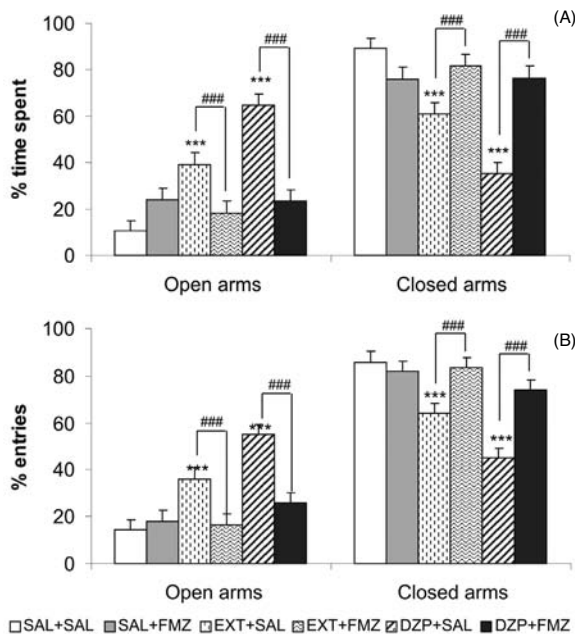
The administration of flumazenil, an antagonist of the benzodiazepine binding site on the GABA$_A$ receptor, completely blocked the exploration increase of the open arms caused by the 300 mg/kg HE extract, as it also did for diazepam (Fig. 3).

Barbiturate Sleeping Time

The oral administration of both extracts (300 mg/kg) resulted in a statistically significant decrease in the sleep latency (Table 1). An increase in the sleeping time was also observed after treating with 300 mg/kg AQ and both doses of HE (300 and 600 mg/kg).

Open Field

At the higher dose (600 mg/kg), HE de-

creased the locomotor activity of the animals, as characterized by a significant decrease in the number of crossings and rearings in the open field test (Table 2). On the other hand, the AQ extract had no effect in this test.

[³H]Flunitrazepam binding

As the effect of 300 mg/kg HE in the Elevated Plus Maze test indicated an anxiolytic effect, we tested this extract in the [³H]flunitrazepam assay. When tested at 10-1000 µg/ml, HE did not modify the binding of [³H]flunitrazepam binding to crude rat brain synaptosomes (data not shown).

DISCUSSION

The present study demonstrated that aqueous (AQ) and hydroethanolic (HE) extracts of *Passiflora alata* although having a similar composition in relation to flavonoids and saponins presented a different pharmacological profile. At tested doses HE presented both anxiolytic and sedative effects while AQ seems to have had

Group	Dose (mg/kg)	Sleep latency (min)	Sleeping time (min)
SAL (=10)	-	4.8 ± 0.6	25.0 (17.0-44.0)
AQ (n=10)	300	3.5 ± 0.8 *	68.0 (62.0-97.75) ###
AQ (n=10)	600	4.5 ± 1.1	45.0 (17.0-52.0)
HE (n=9)	300	3.6 ± 1.0 *	113.0 (98.0-139.5) ###
HE (n=11)	600	3.9 ± 1.0	89.5 (55.0-3.25) ###
DZP (n=10)	2	3.2 ± 0.8 **	97.0 (89.0-120.0) ###

Table 1. Effects of pretreatment with diazepam (DZP), aqueous (AQ) and hydroethanolic (HE) *Passiflora alata* extracts on barbiturate sleeping time (pentobarbital, 40 mg/kg, i.p.). The values of sleep latency and sleeping time are expressed as mean ± S.D. and median with interquartile ranges, respectively. * $p<0,05$ ** $p<0,01$ $(F_{(5,59)}=4,427)$ (ANOVA followed by Student-Newman-Keuls test); ### $p<0,001$ $(H=37,645)$ (Kruskal-Wallis) compared with control group (SAL).

Group	Dose (mg/kg)	Crossings	Rearings	Groomings
SAL (=10)	-	121.3 ± 37.6	27.7 ± 11.0	2.9 ± 2.1
AQ (n=10)	300	151.9 ± 40.8	26.9 ± 10.1	2.7 ± 1.6
AQ (n=8)	600	155.6 ± 37.7	32.2 ± 14.5	3.0 ± 2.7
HE (n=10)	300	128.5 ± 46.6	18.1 ± 13.5	2.0 ± 1.1
HE (n=8)	600	63.6 ± 33.6 **	9.3 ± 9.5 ##	2.4 ± 2.1

Table 2. Effects of treatment with aqueous (AQ) and hidroethanolic (HE) *Passiflora alata* extracts on the open field test. Data are expressed as mean ± S.D. ** $p<0,01$ $(F_{(4,44)}=7,006)$ ## $p<0,01$ $(F_{(4,44)}=7,006)$ (ANOVA followed by Student-Newman-Keuls) compared with control group (SAL).

only hypnotic effects. Similar results were reported for *P. incarnata* extracts [26]. These authors observed that the aqueous extract induced sedative effects while the hydroethanolic extract produced an anxiolytic effect at the same dose (400 mg/kg). More recently, De Castro *et al.* [27] reported an anxiolytic effect for a hydroethanolic extract, but not for an aqueous extract, from *P. quadrangularis*.

The AQ hypnotic effect was dose-dependent, with significant decrease of the pentobarbital sleep latency and increase of the sleeping time at 300 mg/kg (p.o.) but no significant effect at 600 mg/kg, indicating the possibility of a bell-shape dose-effect curve.

With respect to HE, this extract (300 mg/kg, p.o.) increased the plus-maze open arms exploration (time and entries), without causing locomotor activity changes in the Open Field test. These findings point to a specific anxiolytic-like effect, which was blocked by flumazenil (6 mg/kg, i.p.). Such pharmacological profile has also been reported for hydroethanolic extracts from *P. actinia* [28] and *P. incarnata* [10]. However, in concentrations up to 1000 µg/mL, HE did not displace the [³H]flunitrazepam binding to rat brain synaptosomes. A possible explanation for

thispuzzling result could be that *in vivo* biotransformation of some constituent(s) of the extract is necessary for generating active metabolites acting at the benzodiazepine receptor. The metabolism of flavone C-glucosides involves deglycosylation and the openings of the heterocyclic C ring [29]. Based on the pharmacophore models proposed to describe benzodiazepine binding site [30] and also the interaction of flavonoid derivatives with this site [31,32], we can suppose that the sugar moiety linked to C-6 or C-8 on flavone nucleous could impair the *Passiflora* flavonoids (such as vitexin and isovitexin) binding to the benzodiazepine site of $GABA_A$ receptor complex by causing a steric hindrance. These assumptions warrant further studies.

The anxiolytic effect of HE, as for the hypnotic effect of AQ, was also only observed at the lower dose used (300 mg/kg, p.o.). This observation could be related to a sedative effect overlapping the anxiolytic one since HE 600 mg/kg, p.o. also increases the barbiturate sleeping time and reduced the number of crossings and rearings in the open field test. The relation between dose and anxiolytic or sedative effects was already reported for extracts from *P. actinia* [28,33] and *P. incarnata* [9]. This relationship be-

tween the dose and anxiolytic or hypnotic-sedative effect is classic for the benzodiazepines like diazepam [34,35].

The difference of pharmacological profile observed between the two extracts probably reflects differences in the chemical composition as much qualitative as quantitative feature. As detected by thin layer chromatography the hydroethanolic extracts have more lipophilic compounds than aqueous extracts. Thus it is reasonable to suppose that anxiolytic effect is related to the lipophilic substances while the hypnotic-sedative effect observed for both extracts can be due to the hydrophilic molecules. It is also important to consider that the hydroethanolic extract tested in this study differs from the other ones earlier evaluated since it presents a higher alcoholic grade.

In conclusion, the results presented in this work are in agreement with previous reports on the pharmacological activities of the *Passiflora*

genus and does not nullify the popular use of *P. alata* for tranquilizing purposes. The present study also emphasizes the necessity of considering the relation between the kind of effect observed –hypnotic or anxiolytic– and the dose and solvent used for preparing the extracts when manufacturing *Passiflora alata* pharmaceutical products. As a final point we remark that as far we know it was the first time that the *Passiflora* extracts binding to benzodiazepine site was investigated. The results exposed the need for further studies on *Passiflora* pharmacology and chemistry.

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